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A Review of Harnessing RNA Interference (RNAi) and Antisense Technology for Crop Improvement

**James Nnanna Ukwa^{1,*}, Friday Nweke Nwalo², Patrick Maduabuchi Aja¹,
and Mary Oluchi Uda³**

¹ Department of Biotechnology, Ebonyi State University, Abakaliki, Nigeria

² Department of Biotechnology, Alex Ekwueme Federal University, Ndufu-Alike, Nigeria

³ Department of Science Education, Ebonyi State University, Abakaliki, Nigeria

*E-mail address: ukwajamesnnanna24@gmail.com

ABSTRACT

RNA interference (RNAi) is a natural, sequence-specific gene regulation mechanism triggered by double-stranded RNA (dsRNA), leading to translation inhibition or transcriptional repression. Scientists have harnessed RNAi and Antisense RNA technologies to manipulate gene expression in crop plants, offering a new frontier in plant biotechnology. RNAi in particular, has demonstrated greater precision, efficiency, and stability compared to antisense technology approaches. Both technologies have been successfully applied to improve crop traits, including nutritional quality, allergen and toxin reduction, enhanced resistance to biotic and abiotic stresses, morphological changes, male sterility, secondary metabolite production, and seedlessness. While these advancements present promising solutions for crop improvement, they also raise biosafety concerns. Therefore, thorough risk assessments are essential to ensure the safe deployment of genetically modified (GM) crops. This review highlights the applications, benefits, and challenges of RNAi and Antisense technologies in crop enhancement.

Keywords: RNAi, Antisense, GM crops, biosafety, biotic stress, abiotic stress, allergens, gene expression, genome modification

1. INTRODUCTION

In the ongoing effort to combat global food security challenges, scientists have increasingly turned to advanced molecular techniques, specifically RNA interference (RNAi) and antisense technology, as powerful tools for crop improvement. These biotechnological approaches enable precise regulation of gene expression, fostering the development of desirable traits in crop plants. Traditional plant breeding methods, while foundational, are time-consuming, labor-intensive, and face hindrance due to complications such as linkage drag. Moreover, they are proving insufficient in addressing the rapidly growing global population, which currently stands at around 7.7 billion and is projected, according to Rajam, 2020, to reach 9.7 billion by 2050. Over the past two decades, biotechnological approaches and techniques have gained much attention as a more efficient, precise, and reliable alternative for crop-traits improvement (Choudhary *et al.*, 2018). Among the most effective strategies are RNA interference (RNAi) and antisense technology, both of which offer significant potential for sustainable crop production with more infinitesimal environmental impact (Zhang *et al.*, 2017; Alamalakala *et al.*, 2018; Niu *et al.*, 2021; Secic and Kogel, 2021). These biotechnologies involve a multi-step process: identifying target gene(s), designing RNAi constructs or antisense constructs, integrating them into vectors, transforming the plant, and finally screening and evaluating the resulting traits (Bruening and Lyons, 2000).

RNA interference is a naturally occurring, evolutionarily conserved defense mechanism that uses double-stranded RNA (dsRNA) to silence specific genes by degrading messenger RNA (mRNA). It functions both through host-induced and spray-induced gene silencing, to get at pests and pathogens and to regulate plant gene expression (Sang and Kim, 2020). Today, molecular approaches such as molecular breeding, antisense RNA technology, crop gene editing, and genetic transformation are among the most accepted and effective techniques in crop improvement (Afzal *et al.*, 2020; Zaidi *et al.*, 2019).

Genome editing tools like Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and CRISPR/Cas9, have enabled rapid and precise modification of gene functions, leading to the development of crops with enhanced disease resistance (Gaj *et al.*, 2013; Bezie *et al.*, 2020).

The attention of this article is centrally on RNA interference and Antisense technology. These mechanisms function by using small RNAs to interfere with mRNA translation, effectively suppressing gene expression. While small interfering RNAs (siRNAs) guide the degradation of RNA through the RNAi pathway, microRNAs (miRNAs) regulate gene expression by controlling translation (Ferguson, 2020).

In RNAi, the introduction of double-stranded RNA into a eukaryotic cell activates the gene silencing mechanism. While most cellular RNAs are single-stranded and inert, dsRNA with self-complementary regions can initiate silencing (Montgomery *et al.*, 1998; Fire *et al.*, 1998). Gene silencing may also occur through co-suppression or quelling, where dsRNA formed from transgene constructs, consisting of both sense and antisense strands, triggers the RNAi pathway. Specific targeting of mRNA for silencing can be achieved by introducing corresponding dsRNA into the cell (Timmons and Fire, 1998; Wesley *et al.*, 2001). For long-lasting effects, transgenes that produce RNA capable of forming hairpin structures, often referred to as RNAi constructs, are introduced into the plant genome. Another strategy is virus-induced gene silencing, where plant viruses are engineered to carry fragments of target genes. During viral replication, dsRNA regions may form due to self-complementary sequences,

triggering the host's RNA silencing machinery, which targets the viral genome along with the inserted host gene fragment (Ruiz *et al.*, 1998; Ratcliff *et al.*, 2001).

Silenced RNAs are either degraded or prevented from translation. The silencing process is initiated by dsRNA or hairpin-structured RNA (hpRNA), which is recognized by DICER or DICER-like enzymes. These enzymes, along with Argonaute (AGO) proteins, cleave the dsRNA into small RNAs (20–24 nucleotides in length). One strand of the resulting duplex is incorporated into an RNA-Induced Silencing Complex (RISC), which is then guided to a complementary RNA strand. The AGO protein cleaves the target RNA at a central position (usually between nucleotides 10 and 11) of the small RNA (Baulcombe, 2004; Ender and Meister, 2010). Key components of the process include RISC (RNA-induced silencing complex), RISC* (activated RISC), AGO (Argonaute proteins), and RdRP (RNA-dependent RNA polymerase). The specificity of RNA interference depends on the sequence of the dsRNA, making it a highly targeted approach. In plants, animals, and fungi, various RNA silencing mechanisms are now well understood. At least three main RNAi-related mechanisms operate in plants, all initiated by dsRNA that is processed into short silencing RNAs:

- a) Short interfering RNAs (siRNAs) – These degrade complementary mRNA to protect against viruses.
- b) MicroRNAs (miRNAs) – These bind endogenous mRNAs, leading to their degradation or translation inhibition.
- c) DNA-targeting siRNAs – This variant targets DNA itself, affecting transcription rather than post-transcriptional processes (Marie *et al.*, 2023). The discovery and development of RNAi have significantly advanced the fields of genetic engineering and functional genomics, offering transformative potential for global agriculture.

RNAi constructs are typically designed to express self-complementary sequences homologous to the target gene in the form of hairpin RNA (hpRNA). The incorporation of a spacer sequence, usually an intron, between the complementary regions results in intron-hairpin RNA (ihpRNA), which enhances RNAi efficiency (Wesley *et al.*, 2001). Tailoring RNAi constructs for varying degrees and patterns of gene silencing is crucial for genetic engineering applications. For optimal RNAi efficiency in specific plants and applications, the choice of vector, promoter, marker, and transformation method is essential (Saurabh *et al.*, 2014).

Summarily, the initial steps in the biogenesis of miRNAs and siRNAs diverge, producing their respective dsRNA precursors. Subsequently, both types of small RNAs are generated via the cleavage of these dsRNA precursors by Dicer or Dicer-like enzymes, members of the RNase III family of dsRNA-specific endonucleases (Bernstein *et al.*, 2001; Hutvagner *et al.*, 2001). These small non-coding RNAs (miRNAs and siRNAs), in association with RISC, AGO, and other effector proteins, ultimately mediate gene silencing. In genetically transformed plants, the expression of both sense and antisense gene sequences along with a spacer produces ihpRNA. The potential of RNAi as the most efficient tool for genetic engineering to knockdown the gene expression is summarized as its benefits in Table 1 below.

Since the early 21st century, several classes of small non-coding regulatory RNAs have been discovered across various model organisms. MicroRNAs (miRNAs) were first identified in *Caenorhabditis elegans* as regulators of the juvenile-to-adult developmental transition (Lee *et al.* 1993; Reinhart *et al.* 2000). Subsequently, they were found to play a broader role in regulating multiple developmental processes in plants such as *Arabidopsis thaliana* and *Zea mays* (Wu and Poethig 2006; Wu *et al.* 2009; Aukerman and Sakai 2003; Chuck *et al.* 2007a,

2007b). Gene silencing through RNA interference (RNAi) can be initiated by either long double-stranded RNA (dsRNA) or short-hairpin RNA (shRNA) precursors, both of which share sequence homology with the gene targeted for silencing (Fire 1999). The presence of long dsRNA, whether introduced via a transgene, derived from rogue genetic elements (e.g., transposable or repetitive elements), or from viral sources, triggers the RNAi pathway by recruiting the Dicer enzyme (Bernstein *et al.* 2001). Dicer cleaves these dsRNA molecules into short fragments, approximately 21–25 base pairs in length, known as small interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999).

Table 1. Benefits of RNAi for gene silencing in plants.

a) Precise: No off – target reported. But may affect the feedback loops.
b) Efficient: CSIRO plant Industry has reported that 70 – 100% of transformed plant show the silencing phenotype.
c) User friendly: Employs several user – friendly tools available to perform RNAi in plants.
d) High throughput: High – throughput vectors are designed to make an hpRNAi construct
e) Stable: hpRNAi has been shown to be stably inherited over several generations
f.) Flexible: Individual or multiple genes can be silenced with single construct. Allen <i>et al.</i> (2004) have shown effective silencing of two genes from one hpRNAi construct.
g) Better than antisense: the highest silencing achieved with antisense construct has been reported by Wesley <i>et al.</i> 2001, as being as good as the least silenced plant with hpRNAi.

Antisense technology on the other hand, is among the most established approaches for silencing specific genes; and it holds wide-ranging applications in crop improvement, treatment of genetic disorders, viral resistance, and gene regulation. In this method, the sense strand refers to the 5' to 3' mRNA or DNA molecule, while its complementary strand is termed the antisense strand. Antisense molecules function by hybridizing to the corresponding sense strand via hydrogen bonding. The resulting double-stranded complex is recognized as foreign and targeted for degradation.

Although DNA can also be used in antisense strategies, resulting in the formation of a triplex helix (Rakoczy, 2001), RNA antisense strands, typically around 13 bases in length, are more effective. These may be either catalytic (ribozymes) or non-catalytic. Ribozymes cleave specific RNA targets, while non-catalytic antisense RNAs inhibit RNA processing and are more widely used than their DNA counterparts. Antisense molecules can interfere with gene expression at three cellular stages. The first is transcription, although current evidence for antisense interference at this stage is limited. The second stage involves RNA processing. When an antisense strand binds its sense counterpart, a duplex is formed; however, RNA–RNA duplexes are inherently unstable and prone to rapid degradation by nucleases.

This instability may explain the lack of direct evidence for duplex formation, as the hybrid degrades quickly, rendering the mRNA unavailable for translation. The third and most prominent stage is translation inhibition. Here, antisense strands compete with ribosomes for binding to the sense RNA. Effective inhibition requires that the antisense strand binds the target mRNA before the ribosome can initiate translation. To construct antisense RNA, the orientation of the gene is reversed relative to its promoter, ensuring that the antisense strand is transcribed. This antisense RNA binds to the target mRNA and activates RNase H, an enzyme that degrades the mRNA, thereby preventing the production of the encoded protein.

The antisense technology is based on the principle of inserting a gene in reverse orientation into a vector. Since the mechanism obstructs translation, it is stoichiometric, preventing the synthesis of the targeted gene product. The process involves hybridization between sense and antisense RNA strands to form double-stranded RNA, which is rapidly degraded by ribonucleases, blocking gene expression. Additionally, antisense RNA may prevent ribosomal binding to the sense strand, further inhibiting translation. Simply put, the introduction of a complementary oligonucleotide into a cell can bind a specific mRNA to form a cytoplasmic RNA dimer. This prevents access to the ribosome, and the dimer is subsequently degraded by ribonucleases, effectively silencing the gene.

Mechanism of Antisense Technology

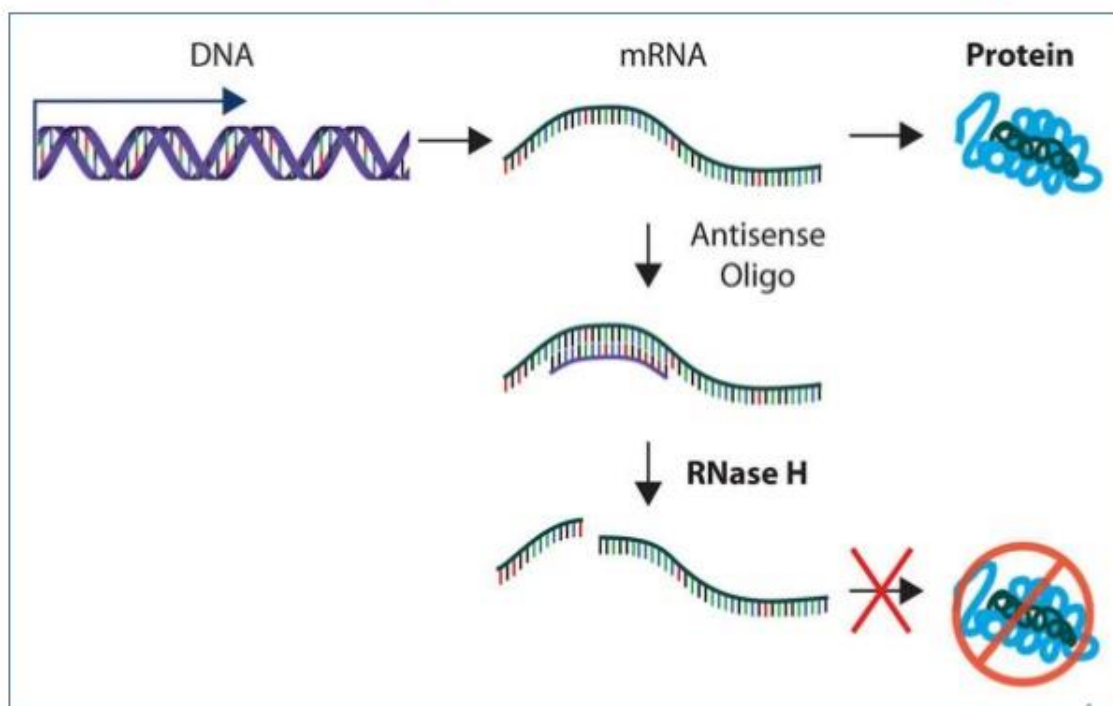


Fig. 1. Schematic representation of the basic mechanism of Antisense technology.

RNAi when compared with Antisense technology reveals that it is a more complex process, as shown in table 2 below.

Table 2. RNAi vs Antisense.

RNAi	ANTISENSE
RNAi technology involves degradation of mRNA by small interfering RNAs triggering catalytic gene silencing.	In Antisense technology mRNA is degraded by RNase H.
Works in a little bit more complex manner, undergoing several procedures, producing proteins and RISC.	It is much basic in terms of the number of nucleotide sequence required, about 20 nucleotides maximum and antisense can easily bind that and degraded by RNase H, an endonuclease.
Requires specific protein for enzyme to degrade, e.g. DICER	RNase is unspecific nuclease kind of enzyme.
RNAi depends on the template RNA which are much longer twice compared to (15 – 20) of Antisense.	A few numbers of templates can be easily introduced into the organism of choice. Prevention by Antisense is to block protein synthesis via mRNA degradation. The problem is as it targets mRNA, there are many copies of mRNA produced from specific gene; hence high concentration of Antisense is required.

(Wesley *et al.*, 2001).

Antisense RNA can be classified into three categories based on their regulatory mechanisms: **Class I** antisense RNAs are directly complementary to the coding regions of the target mRNA. Their binding to the target mRNA leads to either its destabilization or direct inhibition of translation. **Class II** antisense RNAs bind to the non-coding regions of the target RNA, causing indirect effects. These include the formation of alternative secondary structures that sequester or obscure the ribosome binding sites, thereby inhibiting translation. **Class III** antisense RNAs regulate transcription of the target mRNA through mechanisms similar to transcriptional attenuation.

A clearer distinction between RNA interference (RNAi) and Antisense technology lies in their mechanisms of action. RNAi can be triggered either exogenously by introducing double-stranded RNA (dsRNA) or endogenously, to regulate gene expression. In contrast, antisense technology involves the artificial introduction of a single-stranded antisense RNA that targets the complementary sense strand already present in the target cell. In RNAi, both sense and antisense strands are introduced as dsRNA, whereas in antisense technology, only the antisense strand is introduced.

Additionally, RNAi is considered a more complex process. Antisense technology, on the other hand, is more direct and comparatively simpler. RNAi has been shown to be more effective than antisense technology. For instance, the highest level of gene silencing achieved using antisense constructs was equivalent to the lowest silencing level observed with hairpin RNAi (hpRNAi) (Wesley *et al.*, 2001). RNAi achieves gene silencing by mRNA degradation

through small interfering RNAs (siRNAs), which initiate catalytic gene silencing. Moreover, RNAi utilizes template RNAs that are significantly longer than the 15–20 nucleotide sequences typically used in antisense technology (Cheryl, 2016). Despite these differences, both RNAi and antisense technologies serve the same ultimate purpose, gene expression inhibition, and are widely used in reverse genetics for studying gene function.

2. APPLICATIONS OF ANTISENSE AND RNAi TECHNOLOGY

2. 1. Antisense Technology in Crop Improvement

Antisense RNA and RNA interference techniques are extensively used to develop crops with desirable traits such as resistance to insects, nematodes, and various pathogens (Ali, Datta and Datta, 2010; Auer and Frederick, 2009). These small RNA technologies have contributed to a paradigm shift from the traditional “one gene–one protein” concept, highlighting the significant role even non-coding DNA can play in cellular function and organism development (Auer and Frederick, 2009).

2. 1. 1. Extended Shelf Life (Flavr Savr Tomato)

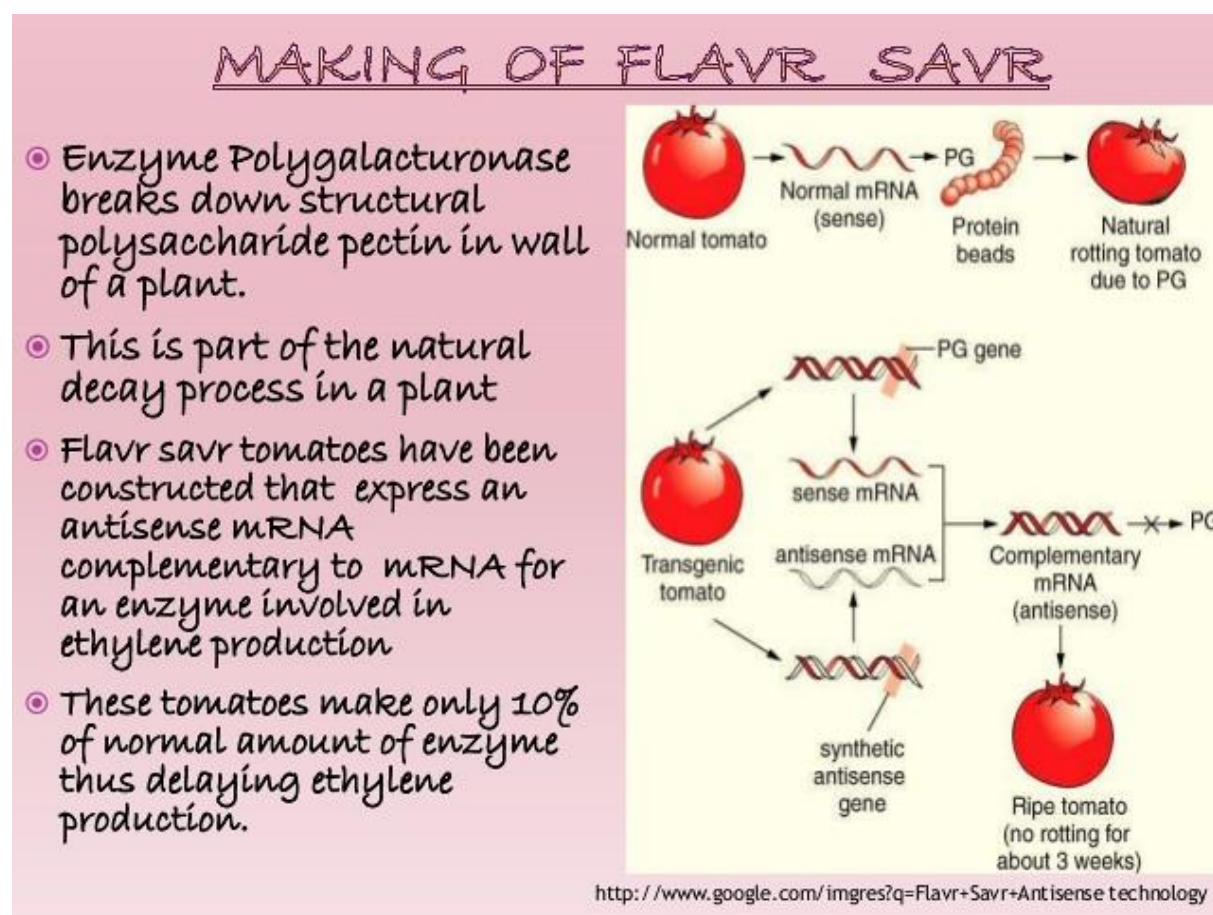


Fig. 2. Enhanced shelflife: the making of Flavr Savr Tomato
<http://www.google.com/imgres?q=Flavr+Savr+Antisense+technology>

Both antisense technology and RNA interference have been used to enhance the shelf life of crops by suppressing genes associated with spoilage. This advancement helps reduce food waste and ensures a consistent supply of fresh produce. A prominent example is the development of the Flavr Savr tomato, which was achieved through antisense inhibition of the polygalacturonase (PG) gene. This gene encodes an enzyme responsible for the degradation of the cell wall during fruit ripening. By inhibiting this enzyme, the shelf life of the tomato was significantly improved. Antisense technology has also been applied to potato crops to reduce tuber discoloration after bruising (Kramer and Redenbaugh, 1994; Burning and Lyons, 2000).

2. 1. 2. Artificial Antisense RNA Regulation of Gene Expression

Artificial modulation of gene expression using antisense RNA has been demonstrated in both plants and animals. To optimize conditions for effective antisense RNA-mediated regulation, researchers targeted various lengths and regions of the sense gene for β -galactosidase. Key findings from the study include:

- 1) Effective antisense RNA must be complementary to the 5' region of the sense mRNA and contain a functional ribosome binding site at the 5' end.
- 2) The molar ratio of antisense to sense RNA typically ranges from 60:1 to 600:1.
- 3) In rare cases, a 1:1 molar ratio may be sufficient (Rehman *et al.*, 2024).

2. 1. 3. Virus Resistance via Antisense RNA

Antisense RNA technology has also been employed to reduce viral accumulation in plants. Notable examples include effective resistance against potato virus X (PVX), tobacco mosaic virus (TMV), and cucumber mosaic virus (CMV).

2. 2. Applications of RNAi Technology in Crop Improvement

2. 2. 1. Plant Pathogen and Pest Control

Gene silencing of plant pathogens and pests has shown promise in agricultural biotechnology. For example, researchers developed an RNAi-based plasmid containing an interference cassette that generates dsRNAs targeting a novel v-ATPase transcript in whitefly (*Bemisia tabaci*), a major pest in tropical and subtropical regions. This technology paves the way for creating commercial whitefly-resistant crops, contributing to sustainable agriculture.

2. 2. 2. Seedless Fruit Development

Phytohormones are crucial in regulating flowering, fertilization, and fruiting. Parthenocarpy is valuable when pollination is compromised by extreme conditions, ensuring stable yields. Seedless fruits often exhibit improved texture and shelf life, as observed in watermelon and eggplant (Pandolfini, 2009). In watermelon, seeds are a primary source of fruit deterioration, and replacing them with edible tissue enhances value for consumers and the food industry (Varoquaux *et al.*, 2000).

2. 2. 3. Male Sterility and Fertility Restoration

Male sterility is a vital trait for hybrid seed production. Both conventional and genetic engineering approaches, including RNA interference, have been employed to induce pollen

abortion in crops like tobacco and tomato. RNAi can also restore fertility in male-sterile plants (Nizampatnam and Kumar, 2011; Meena *et al.*, 2017).

2. 2. 4. Biofortification

More than two-thirds of the global population lacks essential dietary minerals (White and Broadley, 2009). RNAi technology enables biofortification of crops like tomatoes with antioxidants (Niggeweg *et al.*, 2004) and essential minerals such as Zn, Mg, Cu, Se, and Fe. This can be achieved through genetic engineering and transient expression systems like agro-infiltration and virus infection (Obembe *et al.*, 2011). Molecular pharming, focused on producing therapeutic compounds and nutraceuticals, holds significant promise.

2. 2. 5. Allergen and Toxin Elimination

Food allergies, often triggered by common foods such as peanuts and mangoes, can be mitigated through RNAi by silencing genes responsible for allergen synthesis. For instance, cassava plants have been modified to reduce linamarin content (Siritungam and Sayre, 2003). Moreso, RNAi has been used to suppress cytochrome P450 enzymes in cassava, significantly reducing cyanogenic glucoside levels (Jørgensen *et al.*, 2005).

2. 2. 6. Altered Phenotype

Trait modification in ornamental plants, such as altering flower color, shape, and plant architecture, has long been practiced. Such changes can also enhance crop yield, as in the case of dwarf rice (Montgomery *et al.*, 1998).

2. 2. 7. Altered Flower Colour and Scent

With growing demand for ornamental flowers, RNAi has been used to modify flower color and scent. Studies showed that RNAi-induced reduction of polyacylated anthocyanins alters flower pigmentation (Seitz *et al.*, 2007; Nakatsuka *et al.*, 2010).

2. 2. 8. Defence Improvement

RNAi has progressed from greenhouse research to field applications, revealing its potential in improving plant defense mechanisms against biotic stressors such as pests and diseases (Hollomon, 2012).

2. 2. 9. Parasitic Weed Resistance

Parasitic weeds significantly reduce crop yields. Conventional control methods have limitations, but RNAi technology offers a promising alternative. Maize variety resistant to *Striga asiatica* was developed using hpRNA-mediated RNAi (de Framond *et al.*, 2007; Yoder *et al.*, 2009).

2. 3. Insect and Nematode Resistance

Insects cause major agricultural losses. RNAi targeting insect-specific genes provides an eco-friendly pest control solution (Ferry *et al.*, 2006; Gordon and Waterhouse, 2007). Similarly, nematode resistance has been achieved by expressing dsRNAs in host plants that silence

parasitism genes (Gheysen and Vanholme, 2007). Huang *et al.* (2006) showed cross-species resistance by targeting a parasitism gene. The *Meloidogyne hapla* genome offers new RNAi targets (Opperman *et al.*, 2008).

2. 3. 1. Virus Resistance

Pathogen-derived resistance (PDR) is a highly effective strategy for virus-resistant crops (Simón-Mateo and García, 2011). Additionally, artificial miRNAs targeting multiple viral gene regions confer broad-spectrum resistance against tospoviruses in tomatoes (Bucher *et al.*, 2006).

2. 3. 2. Bacterial Resistance

Fast-spreading bacterial diseases require preventative strategies. In *Arabidopsis thaliana*, silencing bacterial genes *iaaM* and *ipt* drastically reduced crown gall formation (Escobar *et al.*, 2001; Dunoyer *et al.*, 2006).

2. 3. 3. Fungal Resistance

RNAi-enhanced crops have shown resistance to fungi. For instance, 24 miRNAs responsive to powdery mildew in wheat were identified, regulating 149 potential target genes (Xin *et al.*, 2010).

2. 3. 4. Abiotic Stress Tolerance

Initially, transgenic crops targeted pest and herbicide resistance (Shewry *et al.*, 2008). miRNAs now play broader roles in nutrient balance, development, and abiotic stress responses (Xin *et al.*, 2010). Genome-wide miRNA identification via high-throughput sequencing is key to understanding their regulatory functions (Wang *et al.*, 2011b).

2. 3. 5. Biotic Stress Resistance

RNAi, antisense RNA, and lncRNA technologies help mitigate crop losses from viruses, bacteria, fungi, nematodes, and insects by improving plant immunity (Xin Zhang *et al.*, 2018).

3. FUTURE PROSPECTS

With global food demands rising due to population growth projected at 9.7 billion by 2050 (Rajam, 2020), sustainable agricultural development is imperative (Saurabh *et al.*, 2014; Molesini *et al.*, 2012). Climate change exacerbates these challenges (Jin Xu *et al.*, 2019), but metabolic and genetic engineering offer viable solutions. Carbohydrates and lignin can yield valuable intermediates (Cherubini, 2010), and lipids can provide omega-3 fatty acids (Octave and Thomas, 2009). RNAi can redirect plant metabolic pathways toward beneficial products and away from harmful compounds, improving nutraceutical content (Small, 2007; Vaucheret, 2006; Martino-Catt and Sachs, 2008; Newell-McGloughlin, 2008). RNAi may also help inhibit photorespiration in C3 plants, delay senescence, or induce early flowering. Emerging gene-editing tools such as ZFNs, TALENs, and CRISPR-Cas systems are revolutionizing crop improvement (Bezie *et al.*, 2020; Afzal *et al.*, 2020; Zaidi *et al.*, 2019).

4. CONCLUSION

Malnutrition affects millions globally, especially in developing nations (WHO, 2000; FAO, 2000). Biofortified crops enriched with essential nutrients can address this problem. Developing stress-tolerant, pest- and disease-resistant plants is crucial for food security. Despite concerns regarding antibiotic markers and biosafety, RNAi and antisense technologies offer transformative potential. Comprehensive risk assessments are necessary to ensure safe application (Ravi and Singh *et al.*, 2020). Ultimately, RNAi and antisense RNA technologies represent powerful tools in functional genomics and crop biotechnology (Li *et al.*, 2008a), with the capacity to significantly boost global agricultural productivity.

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